Morbillivirus and cetaceans, a continuously evolving relationship

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Abstract

Following the two *Dolphin Morbillivirus* (DMV) epidemics occurred in 1990-'92 and in 2006-2008 among Mediterranean Sea striped dolphins (*Stenella coeruleoalba*), a number of morbilliviral infection (MI) cases with peculiar features were reported in striped dolphins found stranded along the Spanish coastline. Affected cetaceans showed a subacute-to-chronic, non-suppurative encephalitis, with brain lesions strongly resembling those found in the course of human "*subacute sclerosing panencephalitis*" and "*old dog encephalitis*". Interestingly, brain was the only tissue in which morbilliviral antigen and/or genome could be detected in these dolphins. Beside the peculiar case of morbilliviral encephalitis described in a young striped dolphin found beached in 2009 along the Latium sea coast of Italy, we observed 5 additional MI cases in 2 striped dolphins, 1 bottlenose dolphin (*Tursiops truncatus*) and 2 fin whales (*Balaenoptera physalus*), all of which found stranded in 2011 along the Italian coastline. Noteworthy, 3 out of these cetaceans (2 striped dolphins and 1 bottlenose dolphin) showed immunohistochemical (IHC) and/or biomolecular (PCR) evidence of morbilliviral antigen and/or genome exclusively in their brain, with 1 striped dolphin and 1 bottlenose dolphin also exhibiting a non-suppurative encephalitis. Furthermore, simultaneous IHC and PCR evidence of a *Toxoplasma gondii* coinfection was found in 1 stranded fin whale.

In conclusion, the above results are strongly reminiscent of those reported in striped dolphins after the two MI epidemics occurred in 1990-'92 and in 2006-2008, with evidence of morbilliviral antigen and/or genome being found exclusively in the brain tissue from affected animals.

Introduction

Morbilliviruses have been recognized, for at least 25 years, as biological disease agents of great concern for free-ranging cetaceans (Van Bressem *et al.*, 2009). Before the end of 2006, a morbilliviral epidemic was reported in pilot whales (*Globicephala melas*) around the Strait of Gibraltar (Fernández *et al.*, 2008) and, in the following months, in striped dolphins (*Stenella coeruleoalba*) and pilot whales along the Spanish Mediterranean coast (Raga *et al.*, 2008). Apart from its milder mortality rate, this outbreak shared many similarities with the mass die-off of striped dolphins occurred between 1990 and 1992 in the Mediterranean Sea and caused by a newly identified agent, *Dolphin Morbillivirus* (DMV) (Domingo *et al.*, 1990, 1992; Barrett *et al.*, 1995; Kennedy, 1998; Di Guardo *et al.*, 2005, 2011c; Raga *et al.*, 2008; Van Bressem *et al.*, 2009). More in detail, the virus responsible for the 2006-2008 mortality episodes in pilot whales and striped dolphins showed a close genetic proximity to the DMV strain causing the dramatic die-off which took place 15 years before in the same area (Fernández *et al.*, 2008; Raga *et al.*, 2008; Van Bressem *et al.*, 2009; Bellière *et al.*, 2011). Direct evidence of morbilliviral infection (MI) has been recently reported in several striped dolphins, a pilot whale and a bottlenose dolphin (*Tursiops truncatus*) found stranded on the French Mediterranean coast (Keck *et al.*, 2010), as well as in a striped dolphin (Di Guardo *et al.*, 2011a) and in a fin whale (*Balaenoptera physalus*) beached along the Tyrrhenian Sea coast of Italy, the latter of which also showed a *Toxoplasma gondii* coinfection (Mazzariol *et al.*, 2012).

As already reported after the DMV epidemic occurred between 1990 and 1992 (Domingo *et al.*, 1995), a number of MI cases with peculiar features were also described in striped dolphins found stranded along the coasts of Spain (Soto *et al.*, 2011a, 2011b) and Italy (Di Guardo *et al.*, 2011a). Affected cetaceans showed a subacute-to-chronic, non-suppurative encephalitis, with brain lesions in Spanish dolphins strongly resembling those found in the course of human "*subacute sclerosing panencephalitis*" (SSPE) and "*old dog encephalitis*" (ODE) (Domingo *et al.*, 1995; Soto *et al.*, 2011a, 2011b). Interestingly, brain was the only tissue in which morbilliviral antigen and/or genome could be detected in all these cases (Domingo *et al.*, 1995; Soto *et al.*, 2011a, 2011b; Di Guardo *et al.*, 2011a).

On the basis of what above, the present work was aimed at investigating the pathologic, immunohistochemical and biomolecular features of MI in cetaceans found stranded along the Italian coastline after the 2006-2008 epidemic.

Materials and methods

We investigated a total number of 6 cetaceans found stranded along the coast of Italy between 2009 and 2011. Three out of these animals were striped dolphins (1 male calf, 1 adult male and 1 adult female), 1 was an adult male bottlenose dolphin and 2 were fin whales (1 adult male and 1 subadult female). Apart from the striped dolphin's calf (stranded in November 2009), all the other cetaceans included in this study were found beached ashore during 2011, with 2 of them being live-stranded (the bottlenose dolphin and the female whale). More in detail, the northern Tyrrhenian (adult male fin whale), the central Tyrrhenian (striped dolphin's calf, adult female striped dolphin and adult male bottlenose dolphin), the southern Tyrrhenian Sea (adult male striped dolphin) and the northern coast of Sardinia (subadult female fin whale) were the animals' stranding sites. Details of the 6 cetaceans investigated herein, including their decomposition codes (Geraci and Lounsbury, 2005) and stranding sites, are shown in Table 1.

A detailed *post mortem* examination was carried out on the 6 stranded cetaceans, with representative samples from their organs and tissues being promptly fixed in 10% neutral buffered formalin for histopathological investigations. Portions of the same organs and tissues were additionally frozen at -20° C for parasitological and microbiological studies, as well as for ecotoxicological investigations (not dealt with in the present article). A*d hoc* biomolecular investigations for *Morbillivirus* and *T. gondii* were performed, respectively by means of RT-PCR (Frisk *et al*., 1999) and PCR techniques (Di Guardo *et al*., 2011b; Mazzariol *et al*., 2012), on selected tissue specimens (brain, lung, spleen, mesenteric and pulmonary lymph nodes, heart and skeletal muscle). The specificity of biomolecular results for both pathogens was further confirmed by means of a nested PCR technique (amplifying an internal fragment of the *Morbillivirus* nucleoprotein gene template previously obtained by RT-PCR), along with sequencing and RFLP analysis (Mazzariol *et al*., 2012).

Parallel immunohistochemical (IHC) investigations for *Morbillivirus* and *T. gondii* were also carried out on the same tissues from all the animals under study, utilizing a commercially available monoclonal antibody (MoAb) against the nucleoprotein (N) antigen of *Canine Distemper Virus* (CDV) and a commercially available goat polyclonal Ab against *T gondii*, respectively (*VMRD Inc*, Pullman, WA, USA) (Di Guardo *et al*., 2010; Mazzariol *et al*., 2012).

Finally, *ad hoc* serological investigations against *Morbillivirus*, *T. gondii* and *Brucella* spp. were also performed, whenever possible, on suitable serum samples obtained by centrifugation (at 1,000 to 1,500 revolutions per min for 15 min) of blood clots freshly collected from the heart chambers and/or major vessels, as reported elsewhere (Dubey *et al*., 2005; Di Guardo *et al*., 2010).

Results

The nutritional status and the main anatomo-histopathological findings observed in the 6 stranded cetaceans under study are reported in Table 2. In this respect, it is worth mentioning that 3 animals (the striped dolphin's calf, the bottlenose dolphin and the subadult female fin whale) showed microscopic evidence of a multifocal, non-suppurative meningo-encephalitis, with multinucleate syncytia being occasionally found scattered throughout the brain lesions in the striped dolphin's calf, as well as in prescapular and pulmonary lymph nodes from the subadult fin whale. Furthermore, a coagulase+ *Staphylococcus aureus* strain was recovered from the brain, lung, liver and kidney of the bottlenose dolphin, which also exhibited a suppurative meningitis and choroiditis, along with a suppurative-necrotic broncho-pneumonia and a suppurative nephritis (Table 2).

As far as biomolecular and IHC investigations for *Morbillivirus* and *T. gondii* are specifically concerned, their results are shown in Table 3. More in detail, beside the peculiar case of morbilliviral encephalitis described in the young striped dolphin found beached in 2009 (Di Guardo *et al*., 2011a), we observed 3 additional MI cases in which affected cetaceans (2 striped dolphins and the bottlenose dolphin) showed IHC and RT-PCR evidence of morbilliviral antigen and/or genome exclusively in their brain. Furthermore, simultaneous IHC and PCR evidence of a *T. gondii* coinfection was found in the adult fin whale, as previously reported elsewhere (Mazzariol *et al*., 2012).

Serological investigations against *Morbillivirus* yielded positive results only in the young striped dolphin found stranded in 2009, with a 1:10 virus neutralization (VN) titre being detected in this animal. Furthermore, we obtained no evidence of anti-*Brucella* spp. Abs in all sera examined, with anti-*T. gondii* Abs being also found in the adult female striped dolphin as well as in the bottlenose dolphin, in which 1:5,120 and 1:640 positive titres were observed, respectively.

Discussion

On the basis of the results presented herein, we can affirm that MI continues to represent a reason of concern for cetaceans living in the Mediterranean Sea area, with species other than the striped dolphin – such as the bottlenose dolphin and the fin whale – being still threatened by and succumbing to this infection. Indeed, the pathogenic potential of morbilliviruses for aquatic mammals in general, as well as for cetaceans in particular, is well documented by a relevant number of scientific publications, with striped dolphins and pilot whales being, jointly or individually, the species most dramatically involved in the two major epidemics occurred in the Mediterranean basin during 1990-1992 and 2006-2008, respectively (Domingo *et al*., 1990, 1992; Barrett *et al*., 1995; Kennedy, 1998; Di Guardo *et al*., 2005, 2011c; Fernández *et al*., 2008; Raga *et al*., 2008; Van Bressem *et al*., 2009).

Noteworthy, 3 out of the 6 *Morbillivirus*-infected cetaceans investigated herein showed simultaneous evidence of a *T. gondii* infection, which was diagnosed either directly (as in the adult male fin whale) or indirectly (as in the adult female striped dolphin and in the bottlenose dolphin), with high anti-*T. gondii* Ab titres being detected in the latter two animals. In this respect, it should be also emphasized that coinfection with *T. gondii*, an opportunistic pathogen for cetaceans (Van Bressem *et al*., 2009; Di Guardo *et al*., 2011c), was reported in several DMV-infected striped dolphins during the dramatic morbilliviral epidemic in the Mediterranean Sea from 1990 to 1992 (Domingo *et al*., 1992; Kennedy, 1998). Although the strong immunosuppressive potential displayed by morbilliviruses in aquatic mammals may well explain the occurrence of a *T. gondii* coinfection in the cetaceans investigated herein, it should be additionally underscored that the high tissue concentrations of immunotoxic environmental pollutants - such as organochlorinated contaminants - measured in our DMV and *T. gondii*-coinfected adult fin whale may have caused an even more dramatic immune response impairment in this host (Mazzariol *et al*., 2012). Notwithstanding what above, there are also instances in which *T. gondii* does not apparently behave as an opportunistic but rather as a primary pathogen in free-ranging cetaceans, as recently reported by our group in striped dolphins found stranded along the Ligurian Sea coast of Italy between 2007 and 2008, in which *T. gondii* occurrence was immunohistochemically and biomolecularly confirmed in the brain tissue from 3 animals affected by a multifocal, non-suppurative meningo-encephalitis (Di Guardo *et al*., 2010, 2011b).

In a similar manner, also the generalized infection by a coagulase+ strain of *S. aureus* in the bottlenose dolphin, along with that by *Klebsiella* spp. in the subadult fin whale included in this survey, may be convincingly explained as bacterial complications following the primary MI diagnosed in both cetaceans.

Another quite relevant result of the work presented here is that no microbiological nor serological evidence of infections caused by *Brucella* spp., another pathogen of concern for cetaceans (Van Bressem *et al*., 2009; Di Guardo *et al*., 2011c), was obtained from any of the stranded animals under investigation.

One of the most, if not even the most challenging and intriguing feature of the present study, however, refers to the fact that, beside the peculiar case of morbilliviral encephalitis described in the young striped dolphin found beached in 2009 along the Central Tyrrhenian Sea coast of Italy (Di Guardo *et al*., 2011a), we observed 3 additional MI cases in which affected cetaceans (1 adult female and 1 adult male striped dolphin, along with the adult bottlenose dolphin) showed IHC and RT-PCR evidence of morbilliviral antigen and/or genome only in their brain, with the male striped dolphin and the bottlenose dolphin also exhibiting a multifocal, non-suppurative meningo-encephalitis and combined lesions of multifocal, non-suppurative and suppurative meningo-encephalitis, respectively.

In this respect, a series of peculiar MI cases, characterized by IHC evidence of morbilliviral antigen exclusively in the brain parenchyma, had been already described in adult striped dolphins found beached off the coast of Spain after the dramatic DMV epidemic occurred between 1990 and 1992 in the Mediterranean Sea. These animals were found to be affected, at their turn, by a subacute-to-chronic encephalitis closely resembling human SSPE- and canine ODE-associated lesions (Domingo *et al*., 1995). Interestingly, a far more consistent number of cases of an SSPE/ODE-like encephalitis were also reported in striped dolphins found stranded along the Spanish coastline after the 2006-2008 epidemic, once again with IHC and biomolecular (RT-PCR) evidence of *Morbillivirus* antigen and genome exclusively restricted to the brain (Soto *et al*., 2011a, 2011b).

We strongly believe that 4 out of the 6 MI cases investigated herein show remarkable similarities with those described in Spanish striped dolphins (Domingo *et al*., 1995; Soto *et al*., 2011a, 2011b). Indeed, all 3 striped dolphins and the bottlenose dolphin under study showed IHC and/or RT-PCR evidence of morbilliviral antigen and/or genome only in their cerebral tissue. Nevertheless, the striped dolphin's calf had a multifocal, non-suppurative meningo-encephalitis that was not entirely consistent with SSPE- and ODE-related lesions (Di Guardo *et al*., 2011a), while the bottlenose dolphin exhibited the simultaneous occurrence of a multifocal, non-suppurative meningo-encephalitis, associated with a suppurative meningo-encephalitis most likely resulting from brain colonization on behalf of a coagulase+ *S. aureus* strain. As far as this latter animal is still concerned, it should be also highlighted that, to the best of our knowledge and differently from what reported in striped dolphins (Domingo *et al*., 1995; Soto *et al*., 2011a, 2011b), no other MI cases showing similar neurobiological features have been previously reported in bottlenose dolphins.

A number of different pathogenetic mechanisms have been suggested in order to justify the prolonged viral persistence within the host's central nervous system (CNS), triggering at its turn the development of the typical *Measles Virus* (MV)- and CDV-induced SSPE- and ODE-related lesions in man and dogs, respectively (Rima *et al*., 1987; Summers *et al*., 1995; Cosby *et al*., 2002; Rima and Duprex, 2006). Among such mechanisms, the production of "escape mutants" secondary to the accumulation of point- and hypermutations within virus envelope genes (Reuter and Schneider-Schaulies, 2010) is regarded, along with others, as an elegant "adaptive strategy" providing the viral agent with an extra-capability of "hiding" from host's immune response, whose efficiency is already physiologically reduced in the CNS compartment (Griffin, 2010). This undoubtedly sounds as a very plausible reason behind the fact that no anti-*Morbillivirus* Abs, or a low anti-*Morbillivirus* neutralizing Ab titre (1:10), were respectively detected in the bottlenose dolphin and in the adult female striped dolphin (both of which were found to be infected with DMV), as well as in the striped dolphin's calf showing IHC and/or biomolecular (RT-PCR) evidence of morbilliviral antigen and/or genome exclusively in their brain. Noteworthy, virus persistence within the host's CNS has been recently linked to a selective mutation process involving the fusion (F) and the matrix (M) protein genes of "brain-restricted/confined" morbilliviral isolates recovered from *Phocine*/*Phocid Distemper Virus* (PDV)-infected common seals (*Phoca vitulina*) (Philip Earle *et al*., 2011).

In conclusion, although much work is still needed in order to precisely define the virus- and the host-related biologic determinants underlying the intriguing phenomenon of *Morbillivirus* infection and persistence within the CNS of cetaceans, we cannot yet rule out the hypothesis that a novel, selectively neurotropic morbilliviral strain infected the animals included in the present investigation.

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Table 1: Details of the 6 stranded cetaceans under study.

ID Number	Species	Sex	Age	Decomposition code	Stranding site	Stranding date
1	SD	M	Calf	1	Central Tyrrhenian Sea coast	November 15, 2009
2	SD	F	Adult	1	Central Tyrrhenian Sea coast	June 29, 2011
3	SD	M	Adult	1	Southern Tyrrhenian Sea coast	July 02, 2011
4	BD	M	Adult	0	Central Tyrrhenian Sea coast	June 29, 2011
5	FW	M	Adult	3	Northern Tyrrhenian Sea coast	January 25, 2011
6	FW	F	Subadult	0	Northern coast of Sardinia	October 03, 2011

Footnote: **BD** = Bottlenose dolphin; **FW** = Fin whale; **SD** = Striped dolphin; **F** = Female, **M** = Male.

Table 2: Nutritional status and main anatomo-histopathological findings observed in the 6 stranded cetaceans under study.

ID Number	**Species**	**Nutritional status**	**Meningo-encephalitis (ME)**	**Pneumonia/Broncho-pneumonia (P/BP)**	**Hepatopathy/ Hepatitis (H)**	**Nephropathy/ Nephritis (N)**	**Enteritis (E)**	**Splenitis (S)**	**Lymphadenopathy/ Lymphadenitis (L)**	**Parasitic lesions**
1	SD	Suboptimal	Non-suppurative multifocal ME	Lymphoid interstitial P/Catarrhal BP	Lipidosis	ND	Chronic catarrhal E	ND	ND	Intestinal microgranulomas associated with *Tetrabothrium forsteri*
2	SD	Poor	ND/Coagulase+ *Staphylococcus aureus*	Lymphoid interstitial P/Suppurative BP	ND	ND	ND	Chronic plasmacytic S	Lymphoid depletion and karyorrhexis	ND
3	SD	Suboptimal	ND	Lymphoid interstitial P	ND	ND	ND	ND	Lymphoid depletion	ND
4	BD	Poor	Non-suppurative multifocal ME/Suppurative multifocal ME due to coagulase+ *S. aureus*	Lymphoid interstitial P/Suppurative and necrotic BP due to coagulase+ *S. aureus*	Chronic multifocal H due to coagulase+ *S. aureus*	Suppurative multifocal N due to coagulase+ *S. aureus*	Chronic catarrhal E	ND	Chronic reactive L	Chronic granulomatous panniculitis presumably due to parasitic larval migration/Chronic granulomatous gastritis likely due to *Pholeter gastrophilus*
5	FW	Poor	ND	ND	Massive hepatic congestion	Mild bilateral hydronephrosis	ND	Diffuse splenic congestion	Congestive mesenteric L	Severe infestation by *Pennella* spp. diffusely involving the skin/Protozoan cysts in myocardial and renal tissues/Bilateral hydronephrosis presumably due to *Crassicauda* spp.
6	FW	Poor	Non-suppurative multifocal ME	Non-suppurative bronchiolo-interstitial P/*Klebsiella* spp. isolated from lung	Mild non-suppurative H/*Klebsiella* spp. isolated from liver	Mild chronic interstitial N	Mild catarrhal E	Lymphoid depletion associated with mild congestion/*Klebsiella* spp. isolated from spleen	Marked lymphoid depletion, with occasional syncytia in prescapular and pulmonary lymph nodes	Parasitic *larvae* and eggs of *Crassicauda* spp. in renal pelvis, as well as in blood and lymphatic vessels

Footnote: **BD** = Bottlenose dolphin; **FW** = Fin whale; **SD** = Striped dolphin; **ND** = Not detected.

Table 3: Biomolecular (RT-PCR, PCR), IHC, microbiological and serological findings for *Morbillivirus*, *Toxoplasma gondii* and *Brucella* spp. in the 6 stranded cetaceans under study.

ID Number	**Species**	***Morbillivirus***						***T. gondii***				***Brucella* spp.**	
		RT-PCR	**Nested PCR**	**RFLP**	**Sequencing**	**IHC**	**Serology (VN)**	**PCR**	**Sequencing**	**IHC**	**Serology (MAT)**	**Culture**	**Serology (RSA)**
1	SD	Pos (Brain)	ND	ND	ND	Pos (Brain)	1:10	ND	ND	ND	Neg	Neg	Neg
2	SD	Pos (Brain)	Pos	ND	ND	Neg	Neg	ND	ND	ND	1:640	Neg	Neg
3	SD	Pos (Brain)	Pos	ND	DMV	Pos (Brain)	ND	ND	ND	ND	ND	Neg	ND
4	BD	Pos (Brain)	Pos	ND	ND	Neg	Neg	Neg	ND	Neg	1:5,120	Neg	Neg
5	FW	Pos (Spleen, Liver, Lung)	ND	Pos	DMV	Neg	ND	Pos (Kidney, Heart, Skeletal muscle, Mesenteric lymph nodes)	*T. gondii*	Pos	ND	Neg	ND
6	FW	Pos (Liver, Spleen, Lymph nodes, Skeletal muscle)	ND	ND	ND	Neg	ND	Neg	ND	Neg	ND	Neg	ND

Footnote: **BD** = Bottlenose dolphin; **FW** = Fin whale; **SD** = Striped dolphin; **ND** = Not done; **Neg** = Negative; **Pos** = Positive;
MAT = Microagglutination test; **RSA** = Rapid serum agglutination; **VN** = Virus neutralization.